

C1  
cont.  
dependent (middle) and burst (right) autophosphorylation activity of CaMKIIa, GFP-CaMKIIa and GFP-CaMKIIb. The kinase activity of the in vitro translated constructs is shown. Translated GFP alone was included as a control.--

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✓  
Please replace the paragraph on page 5, lines 14-15, with the following rewritten paragraph:

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C2  
--Figure 3D. Line scan profiles of three different dilutions after phorbol ester addition.--

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✓  
Please replace the paragraph on page 5, lines 24-25, with the following rewritten paragraph:

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C3  
--Figure 4. Requirement for more than one CaMKIIb subunit for targeting CaMKIIa/b hetero-oligomers to the actin cytoskeleton.--

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Please replace the paragraph on page 7, lines 3-15, with the following rewritten paragraph:

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C4  
--"Internal structure" as used herein refers to a separate, discreet, identifiable component contained within a cell. The term "structure" as applied to the constituent parts of a cell is known (see, e.g., R. Dyson, Cell Biology: A Molecular Approach, pg 10, (2d ed. 1978)), and the term "internal structure" is intended to exclude external structures such as flagella and

14  
cont.  
pili. Such internal structures are, in general, anatomical structures of the cell in which they are contained. Examples of internal structures include both structures located in the cytosol or cytoplasm outside of the nucleus (also called "cytoplasmic structures"), and structures located within the nucleus (also called "nuclear structures"). The nucleus itself including the nuclear membrane are internal structures. Structures located within the cytoplasm outside of the nucleus are currently preferred. Thus the term "internal structure" is specifically intended to include any non-uniformly distributed cellular component, including proteins, lipids, carbohydrates, nucleic acids, etc., and derivatives thereof.--

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✓  
Please replace the paragraph beginning on page 7, line 16, bridging page 8, line 9, with the following rewritten paragraph:

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15  
--"Library" as used herein refers to a collection of different compounds, typically organic compounds, assembled or gathered together in a form that they can be used together, either simultaneously or serially. The compounds may be small organic compounds or biopolymers, including proteins and peptides. The compounds may be encoded and produced by nucleic acids as intermediates, with the collection of nucleic acids also being referred to as a library. Where a nucleic acid library is used, it may be a random or partially random library, commonly

known as a "combinatorial library" or "combinatorial chemistry library", or it may be a library obtained from a particular cell or organism, such as a genomic library or a cDNA library. Small organic molecules can be produced by combinatorial chemistry techniques as well. Thus in general, such libraries comprise organic compounds, including but not limited to oligomers, non-oligomers, or combinations thereof. Non-oligomers include a wide variety of organic molecules, such as heterocyclics, aromatics, alicyclics, aliphatics and combinations thereof, comprising steroids, antibiotics, enzyme inhibitors, ligands, hormones, drugs, alkaloids, opioids, benzodiazepenes, terpenes, porphyrins, toxins, catalysts, as well as combinations thereof. Oligomers include peptides (that is, oligopeptides) and proteins, oligonucleotides (the term oligonucleotide also referred to simply as "nucleotide, herein) such as DNA and RNA, oligosaccharides, polylipids, polyesters, polyamides, polyurethanes, polyureas, polyethers, poly (phosphorus derivatives) such as phosphates, phosphonates, phosphoramides, phosphonamides, phosphites, phosphinamides, etc., poly (sulfur derivatives) such as sulfones, sulfonates, sulfites, sulfonamides, sulfenamides, etc., where for the phosphorous and sulfur derivatives the indicated heteroatom for the most part will be bonded to C, H, N, O or S, and combinations thereof. See, e.g., U.S. Patent No. 5,565,324 to Still et al., U.S. Patent No. 5,284,514 to Ellman et al., U.S. Patent No. 5,445,934 to Fodor et

C5  
Cont. al. (the disclosures of all United States patents cited herein are to be incorporated herein by reference in their entirety).--

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Please replace the paragraph on page 8, lines 18-23, with the following rewritten paragraph:

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C6 --"Specifically binds" and "specific binding" as used herein include but are not limited to stereospecific binding, electrostatic binding, or hydrophilic binding interactions. Thus, specifically binds and specific binding are exhibited by at least a two or three fold (or two or three times), greater apparent binding affinity between the binding partners as compared to other proteins or binding partners within the cell in which binding is being detected.--

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✓  
Please replace the paragraph on page 9, lines 25-30, with the following rewritten paragraph:

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C7 --If desired, the second heterologous conjugate can further comprise a detectable group, which detectable group is preferably different from the detectable group located on the first heterologous conjugate and fluoresces at a different wavelength therefrom. For example, both detectable groups could be green fluorescent proteins, yet simply different mutants of green fluorescent protein that fluoresce at different wavelengths.--

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✓  
Please replace the paragraph beginning on page 9, line 31, bridging page 10, line 4, with the following rewritten paragraph:

C8  
--Either or both of the heterologous conjugates may be introduced directly in the cell by any suitable means, such as by electroporation or lipofection. In the alternative, when the heterologous conjugates are fusion proteins, a nucleic acid (typically a DNA) may be stably introduced into the cell (for example, as a plasmid), which nucleic acid includes a suitable promoter segment that controls and causes the expression of a nucleic acid encoding the fusion protein. Again, either or both of the fusion proteins may be produced in the cell in this manner.

✓  
Please replace the paragraph on page 10, lines 5-13, with the following rewritten paragraph:

C9  
--Binding events in the instant invention may be direct or indirect binding events. Indirect binding events are those mediated through an intermediate, or bridging, molecule or conjugate. Administration of such a bridge molecule can be a signal to induce translocation (discussed below). For example, the bridging molecule may be a covalent conjugate of FK506 and cyclosporin, to cause the indirect binding of FKBP12 and cyclophilin (both conventionally cytosolic proteins) to one

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cont  
another. Either of the FKBP12 or the cyclophilin can be modified so that it binds to the plasma membrane, such as by lipidating the protein or forming a fusion protein with the transmembrane domain of a transmembrane protein.--

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✓  
Please replace the paragraph beginning on page 14, line 30, bridging page 15, line 18, with the following rewritten paragraph:

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C10  
--Since not only CaMKII $\alpha$  but also CaMKII $\beta$  is a prominent isoform in the central nervous system, the question arises whether CaMKII $\alpha$  and CaMKII $\beta$  have different roles in regulating neuronal functions. Such functional differences between the two isoforms would have a direct impact on our understanding of cell type specific signaling processes since the relative expression of CaMKII $\alpha$  and CaMKII $\beta$  is markedly different in different brain regions and at different developmental stages. For example, the ratios of  $\alpha$  and  $\beta$  subunits are about 3:1 and 1:4 in adult forebrain and cerebellum, respectively, while in 10-day postnatal mice, the forebrain a:b ratio is 1:1 (Miller and Kennedy, 1985). On a structural basis, recombinant CaMKII $\alpha$  as well as purified brain CaMKII have been shown to form oligomers with approximately 8 to 12 subunits (Kanaseki et al., 1991, Bennett et al., 1983). CaMKII $\beta$  and CaMKII $\alpha$  have a similar overall domain organization and corresponding autophosphorylation consensus sequences and

C10  
cont.

even though the calmodulin binding affinity of CaMKII $\beta$  is slightly higher than that of CaMKII $\alpha$  the regulation of different CaMKII isoforms by Ca<sup>2+</sup>/CaM and autophosphorylation is overall similar (Miller and Kennedy, 1985; De Koninck and Schulman, 1998; GuptaRoy and Griffith, 1996). Despite these similarities, it has been controversial whether CaMKII $\beta$  forms oligomers on its own (Yamauchi et al., 1989), whether CaMKII $\alpha$  and CaMKII $\beta$  form hetero-oligomers when expressed at the same time (Kanaseki et al., 1991) and whether the two isoforms are differentially localized within cells (Scholz et al., 1988, Nomura et al., 1997).--

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Please replace the paragraph beginning on page 15, line 28, bridging page 16, line 7, with the following rewritten paragraph:

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C11

--A GFP-based protein-protein interaction assay (Pull-Out binding assay) was then developed to explore the binding interactions between CaMKII $\alpha$  and CaMKII $\beta$  isoforms in living cells. When expressed alone, CaMKIIb was found to form homo-oligomers with an average size that is markedly smaller than the approximately thirteen subunits measured for CaMKIIa homo-oligomers. When expressed at the same time, CaMKIIb isoforms incorporated equally well into either CaMKIIa or CaMKIIb oligomers (and vice versa). Half-maximal targeting of CaMKIIa oligomers to the cytoskeleton was achieved if at least 15% of

C11  
cont. CaMKIIB were present in the same cell, suggesting that a small number of CaMKIIB subunits are required to dock CaMKIIa/b hetero-oligomers with approximately thirteen subunits to F-actin. Our studies suggest that the synaptic localization of CaMKII activity is controlled by the relative expression of CaMKIIB F-actin docking modules.--

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Please replace the paragraph on page 16, lines 11-20, with the following rewritten paragraph:

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C12 --The cDNA for rat CaMKII $\alpha$ ,  $\beta$  and  $\beta'$  were generous gifts from Dr. Howard Schulman. The construction of the GFP-CaMKII $\alpha$  vector was described previously (Shen and Meyer, 1998). To obtain the in vitro transcription vector for CaMKII $\alpha$  without GFP, the CaMKII $\alpha$  cDNA was amplified by PCR and cloned into the in vitro transcription vector dSHiro3. DNA sequencing was performed to exclude PCR errors. GFP-CaMKIIB and CaMKIIB were also cloned into the SHiro3 and dSHiro3 vectors using a similar PCR strategy. The construction of PM-GFP or Cys-GFP was described previously (Oancea et al., 1998). PM-CaMKII $\alpha$  and  $\beta$  were made by replacing the GFP sequence with CaMKII $\alpha$  and  $\beta$  coding sequence in the same SHiro3 vector.--

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Please replace the paragraph on page 17, lines 3-16, with the



following rewritten paragraph:

C13  
--CaMKII autophosphorylation assays were performed as described previously (Hanson et al., 1994). Briefly, CaMKII isoforms and fusion constructs were autophosphorylated at 30°C in 25ml reactions containing 50 mM PIPES (pH 7.0), 10mM MgCl<sub>2</sub>, 500 mM CaCl<sub>2</sub>, 600 nM calmodulin, 50 mg/ml BSA, and 200 mM [γ-<sup>32</sup>P]ATP (6000cpm/pmol). The Ca<sup>2+</sup> dependent autophosphorylation reaction was started by adding in vitro translation product into the reaction mix and stopped by addition of EDTA (16.7 mM final concentration) 30 seconds later. To measure the extent of the Ca<sup>2+</sup>-independent autophosphorylation, a 30 second reaction at high Ca<sup>2+</sup> was followed by a 120 second secondary incubation in the presence of added EGTA (3.3 mM final concentration). In control experiments, 3.3 mM EGTA were included in the initial reaction mix. The reaction mix was then resolved on a SDS-PAGE and subjected to Phosphorimager analysis. The densitometry of bands was measured and corrected by the amount of kinase which was determined in a separate in vitro translation reaction with <sup>35</sup>S-methionine as described above.--

Please replace the paragraph on page 18, lines 2-11 with the following rewritten paragraph:

C14  
--RBL 2H3 cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 20% fetal bovine serum

C14  
cont.  
(Life Technologies, Gaithersburg, MD) at 37°C with 5% CO<sub>2</sub>. The cells were plated at  $5 \times 10^4$  cells/cm<sup>2</sup> on glass coverslips and were allowed to attach to the coverslip for a minimum of 3 hours. Hippocampal neurons obtained from 2 to 4 days postnatal rats were cultured as described in Ryan and Smith (1995) and used 10 days to three weeks after plating. A self-built small volume electroporation device for adherent cells was used for electroporation (Teruel and Meyer, 1997). For the transfection of neurons, modified versions of the device and buffer conditions were used. After transfection, the electroporation buffers were replaced with the same culture medium.

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Please replace the paragraph on page 19, lines 15-18, with the following rewritten paragraph:

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C15  
--The probability of having one or more subunits randomly inserted into a hetero-oligomer is  $[1 - (\text{probability to have no subunit inserted})]$ . The probability of having none inserted is  $(R/(R+1))^N$  with R as the ratio of GFP-CaMKIIa to CaMKIIb and N as the number of subunits.--

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✓  
Please replace the paragraph beginning on page 19, line 21, bridging page 20, line 4, with the following rewritten paragraph:

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C16  
--NIH-3T3, RBL cells, and hippocampal neurons were cultured on glass coverslips and transfected with mRNA encoding GFP-

C16  
cont. CaMKIIB fusion construct. Seven to eight hours after transfection, the cells were fixed for 10 minutes at 4°C with 4% paraformaldehyde in PBS (1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 138 mM NaCl and 2.7 mM KCl [pH 7.4]). NIH-3T3 cells and RBL cells were permeabilized for 5 minutes at 4°C with 0.1 % Triton in PBS. Hippocampal neurons were permeabilized for 10 minutes at 4°C with 0.1 % Triton. For F-actin staining, rhodamine phalloidin (Molecular Probes) was incubated with the cells for 30 min at room temperature at a dilution of 1:300 in PBS. For the staining of postsynaptic densities, hippocampal neurons were incubated with an monolonal PSD-95 antibody (Cat.# 05-428, Upstate Biotechnology, Lake Placid, NY) overnight at 4°C at a dilution of 1:200 and then in secondary Cy3 labelled anti-mouse antibody (Cat.# 115-165-062, Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. The cells were washed three times with PBS and coverslips were mounted onto glass slides using buffered glycerol mounting medium.--

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Please replace the paragraph beginning on page 21, line 19, bridging page 22, line 1, with the following rewritten paragraph:

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C17  
--It was tested more directly whether CaMKIIB has more binding interactions than CaMKIIa by comparing the local fluorescence recovery after photobleaching of GFP-CaMKIIB to that of GFP-CaMKIIa. A 2 µm diameter laser photobleach spot was

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cont.

generated in the cell by a short laser pulse and the fluorescence recovery was monitored by rapid confocal imaging. Consistent with the hypothesis that CaMKIIb but not CaMKIIa undergoes binding interactions, the recovery after photobleaching was significantly more rapid for CaMKIIa compared to that for CaMKIIb. This could be quantitatively shown by a calculated average diffusion coefficient of CaMKIIb that was 5 times lower than that of CaMKIIa (Figure IF, see Procedures above for a description of the analysis). Nevertheless, the binding interactions of CaMKIIb were reversible, since most of the GFP-CaMKIIb fluorescence recovered on the time scale of 15 seconds after the laser bleach pulse. Together, these measurements suggest that CaMKIIa expressed alone is a highly mobile protein that has only limited cytosolic binding interactions, while CaMKIIb is bound in a reversible manner to dendritic and cortical structures.--

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Please replace the paragraph beginning on page 22, line 31, bridging page 23, line 7, with the following rewritten paragraph:

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118

--Since actin is highly enriched in dendritic spines and cell cortex (Fisher et al., 1998; Landis and Reese, 1983, Caceres et al, 1983), it is conceivable that the localization of CaMKIIb to dendritic spines is mediated by a direct or indirect binding interaction of CaMKIIb with F-actin. The possible co-localization of CaMKIIb and F-actin in two model cell lines was tested by

C18  
cont.  
using rhodamine-phalloidin as a marker for polymerized actin (F-actin). In RBL-cells, which have predominant cortical F-actin structures, the cortical rhodamine-phalloidin closely co-localized with GFP-CaMKIIB. The same near complete overlap was also observed in NIH-3T3-cells which are rich in actin stress fibres (not shown). This suggests that the actin cytoskeleton co-localization of CaMKIIB is not cell type specific.--

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✓  
Please replace the paragraph beginning on page 23, line 21, bridging page 24, line 4, with the following rewritten paragraph:

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C19  
--It was then tested whether the co-expression of CaMKIIa and CaMKIIB in the same cell affects their respective localization. An effective co-expression of both isoforms was made possible by using an RNA transfection method. In this approach, a large number of translation competent RNA molecules are directly introduced into the cytosol of adherent cells by microporation (Teruel and Meyer, 1997; Yokoe and Meyer, 1996). Thus, RNA encoding different proteins can be mixed and expressed at a defined ratio within each transfected cell. Strikingly, when GFP-CaMKIIa was expressed together with CaMKIIB (without a GFP-tag) in hippocampal neurons, GFP-CaMKIIa became associated with the same dendritic spine and cortical structures (data not shown). A largely cortical localization of GFP-CaMKIIa was also observed in RBL-cells in the presence of CaMKIIB. In contrast,